UREASE ACTIVITY IN TRYPANORHYNCH CESTODES[1]

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The hydrolysis of urea has been reported as an activity of tissue preparations from a number of invertebrate animals. Urease activities were detected in a lamellibranch (*Mytilus edulis*), a gastropod (*Helix pomatia*), an oligochaete (*"Lumbricus agricola"*), and a decapod crustacean (*Astacus fluviatilis*) by Przylecki (1922); in five species of marine molluscs by Albrecht (1921); in the horseshoe crab (*Limulus polyphemus*) by Loeb and Bodansky (1926, 1927); in the eggs and developing embryos of a sea urchin (*Strongylocentrotus purpuratus*) by Brookbank and Whiteley (1954); in the larvae and larval secretions of a blowfly (*Lucilia sericata*) by Robinson and Baker (1939); in a sipunculid (*Sipunculus nudus*) by Florkin and Duchateau (1942). The latter authors also reported activities in tissues of a crayfish (*"Ecrevisse"*) and a lobster (*"Homard"*) (Florkin and Duchateau, 1943). Brunel (1938) also reported urease activity from *Mytilus edulis,* while Baldwin and Needham (1934) detected activity in the nephridium of *Helix pomatia.*

Several species of parasitic nematodes have been reported to possess a urease. Rogers (1952) demonstrated urease activity in *Nematodirus* spp., and stated that lesser activities were found in *Ascaridia galli* and *Haemonchus contortus.* Savel (1955) studied urease in several tissues of *Ascaris lumbricoides.*

Apparently there have been no previous observations of urease activity in flatworms. Van Grembergen and Pennoit-deCooman (1944) failed to detect activity in a trematode (*Fasciola hepatica*) and two cestode parasites of mammals (*Moniezia benedeni* and *Taenia pisiformis*).

The present paper will show that certain trypanorhynch tapeworms from elasmobranch hosts possess urease of quite remarkable levels of activity.

Materials and Methods

Infected elasmobranches were obtained through the courtesy of the Supply Department of the Marine Biological Laboratory. All the fishes came from a trap in Buzzard's Bay, Massachusetts, and were identified by reference to the paper of Bigelow and Schroeder (1953).

Fishes were killed by blows on the head, and cestodes were immediately collected from the spiral valves. A saline solution composed of 250 m*M* NaCl, 4.4 m*M* KCl, 5.1 m*M* $CaCl_2$, and 2.9 m*M* $MgCl_2$ was used for rinsing and incubating the worms. Following several saline rinses to remove adhering materials, the worms

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were incubated for 2–5 hours at 20° C. Such treatment was found to render the tetraphyllid cestode, *Calliobothrium verticillatum*, free of detectable endogenous urea (Read *et al.*, 1959). Subsequent to this incubation period, worms were blotted on hard filter paper and the desired amount of tissue weighed on a torsion balance.

Homogenates of worm tissue were prepared in TenBroeck tissue grinders of suitable size immersed in a cracked-ice bath. The homogenates were 1:9 (w/v) dilutions of wet tissue, the diluent being an ice-cold solution of 5 m*M* disodium ethylenediamine tetraäcetate (EDTA).

Urease activity of tissue homogenates was assayed by a modification of the method described by Sumner (1955), which measures the ammonia produced from the hydrolysis of urea. A complete reaction mixture consisted of 200 microliters of a urea solution of appropriate concentration, 200 microliters of a 100 m*M* buffer

TABLE I

Urease activities in cestodes of elasmobranchs

Worm species	Host	No. determinations	Tissue activity (mean ±s.e.)
Tetraphyllidea			
Calliobothrium verticillatum	*Mustelus canis*	8	ND
Phyllobothrium lactuca	*Mustelus canis*	4	ND
Phyllobothrium foliatum	*Dasyatis centrura*	4	ND
Onchobothrium pseudo-uncinatum	*Dasyatis centrura*	4	ND
Inermiphyllidium pulvinatum	*Dasyatis centrura*	7	ND
Orygmatobothrium dohrnii	*Carcharias taurus*	4	ND
Disculiceps pileatum	*Carcharinus obscurus*	4	ND
Trypanorhyncha			
Lacistorhynchus tenuis	*Mustelus canis*	8	3,330± 40
Grillotia erinaceus	*Raja ocellata*	7	ND
Pterobothrium lintoni	*Dasyatis centrura*	8	33,290±610

Five-minute incubations at 20° C.
ND: not detected, less than 25 micromoles urea hydrolyzed per gram wet tissue per hour.

solution (phosphate buffer, pH 7.0, unless otherwise indicated), and 100 microliters of 10% tissue homogenate. The reaction was initiated by blowing in the homogenate and stopped by blowing in 200 microliters of 20% trichloroacetic acid (TCA). Control preparations for the assay included (1) untreated homogenate in a reaction mixture lacking urea, (2) heat-inactivated homogenate incubated in the presence of urea, (3) zero-time preparations with TCA added prior to the addition of homogenate, and (4) diluent alone (no tissue) in the presence of urea. The latter preparation never yielded detectable ammonia; the other control preparations gave low (about 25 micrograms of ammonia nitrogen per gram wet tissue), and practically identical, ammonia values. All assay values are reported as corrected for the maximum tissue blank value.

Ammonia produced in the reaction mixtures was liberated by the addition of 1.5 milliliters of a saturated solution of potassium carbonate and captured in 1 N

H_2SO_4 by the microdiffusion method of Seligson and Seligson (1951). Ammonia was determined by the procedure of Lang (1958).

Protein was estimated by a modification of the Lowry method (Lowry *et al.*, 1951) using crystalline bovine serum albumin (Armour and Company) for comparison.

Results are reported as tissue activities or as specific activities, here defined as micromoles urea hydrolyzed per gm. wet tissue weight per hour and micromoles urea hydrolyzed per mgm. protein per hour, respectively, assuming that two micromoles of ammonia recovered are equivalent to one micromole of urea hydrolyzed.

Additional experimental procedures will be described in context.

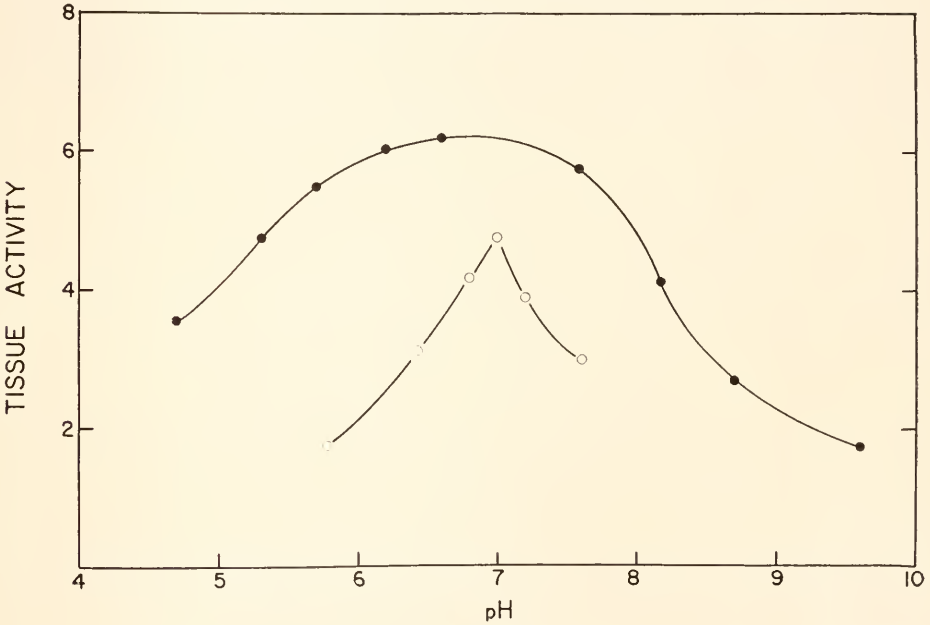

FIGURE 1. Effect of pH on *Lacistorhynchus* urease activity. Tissue activity as micromoles urea hydrolyzed per gram wet tissue per hour in thousands. Open circles with phosphate buffer; closed circles with Tris-maleate. One-minute incubations at 20° C. Each point represents the mean value of four determinations.

RESULTS

A. *Distribution of urease activity among cestodes of elasmobranch fishes*

Read *et al.* (1959) have shown that urea is an important osmotic constituent of certain Tetraphyllidea, a tapeworm group parasitizing elasmobranch fishes; data derived from studies with [^{14}C] urea indicate that in tetraphyllid cestodes urea is a metabolically inactive compound (Simmons *et al.*, 1960).

Ten species of tapeworm from elasmobranches were examined for urease activity by the procedures just described; results of the assays are given in Table I. Of seven tetraphyllids examined, none exhibited activity, while two of three trypanorhynch species examined were quite active in hyrolyzing urea.

Additional *Pterobothrium* material was not forthcoming, and further studies with tissue preparations were made with the more readily available *Lacistorhynchus*.

B. *Studies of Lacistorhynchus urease in tissue homogenates*

Effect of pH on activity

Figure 1 shows activity curves obtained when Sørensen's phospate buffer and tris(dimethyl)aminomethane (Tris)-maleic acid buffer (Gomori, 1955) are employed in the assay.

The very sharp optimum observed at pH 7.0 in phosphate buffer is characteristic also of jackbean urease (Sumner, 1951). Apparently there have been no studies with jackbean urease employing Tris-maleate although Wall and Laidler (1953) found that crystalline jackbean urease showed a marked optimum at pH 8.00 in Tris-sulfuric acid buffer. *Lacistorhynchus* homogenates show no such optimum in Tris-sulfuric acid when the pH is varied between 7.4 and 9.0 (Table II).

TABLE II

Urease activity of Lacistorhynchus homogenates with Tris-H_2SO_4 Buffer

pH	Tissue activity (mean ± s.e.)
7.4	3370 ± 40
7.8	3160 ± 50
8.0	2690 ± 42
8.2	2120 ± 50
8.6	2080 ± 43
9.0	1920 ± 20

One-minute incubations at 20° C.
Activities are mean values of four determinations.

Although the worm activities in Tris-maleate are considerably elevated above those obtained using phosphate buffer, especially on either side of pH 7.0, the latter buffer was employed in subsequent experiments since most studies of urease activities from other sources have employed phosphate buffer.

Activity as a function of enzyme concentration

Linearity of activity as a function of protein concentration was observed over the range examined, 0.068–1.09 mgm. protein (Fig. 2).

Effect of substrate concentration

Table III gives activities of *Lacistorhynchus* homogenates with increasing urea concentrations. The saturating concentration range, observed from about 250 m*M* to 350 m*M* is quite interesting in view of the fact that the urea concentration in the host blood is about 330 m*M* and that of the gut contents is similar (Read *et al.*, 1959).

The worm enzyme may be less susceptible to product inhibition than jackbean enzyme, since the latter exhibits decreasing activities in urea concentrations about

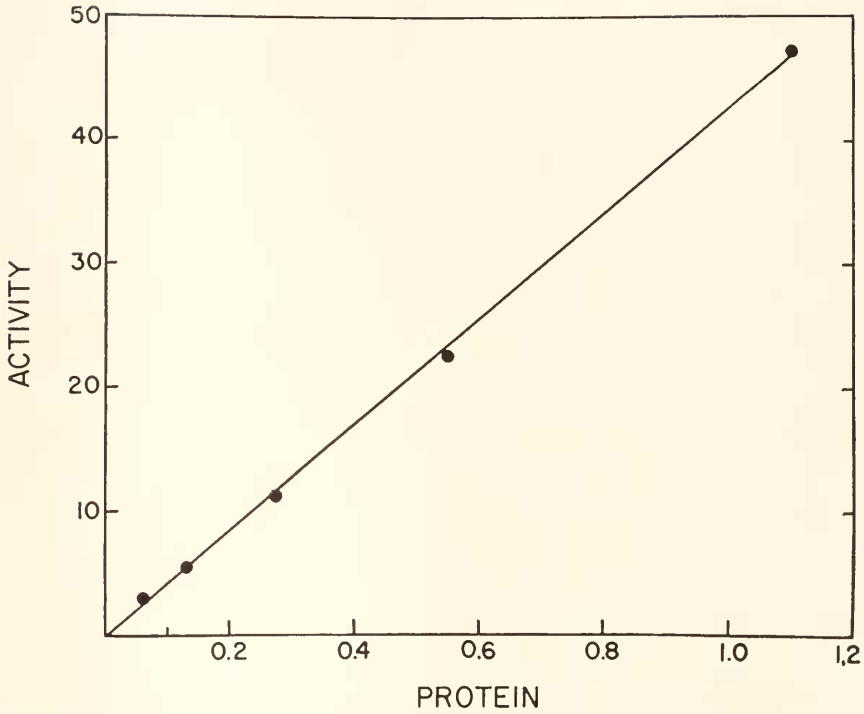

FIGURE 2. Urease activity of *Lacistorhynchus* as a function of protein concentration. One-minute incubations at 20° C. Activity as micromoles urea hydrolyzed per hour. Protein in milligrams. Each point represents the average of duplicate determinations.

250 m*M*–330 m*M*, presumably due to inhibition by the ammonium ions produced (Wall and Laidler, 1953).

Data obtained with the tapeworm urease give good agreement with Michaelis-Menten kinetics when plotted by the method of Lineweaver and Burk (1934), over

TABLE III

Urease activity of Lacistorhynchus homogenates with varying urea concentrations

Urea concentration m*M*	Tissue activity
6.25	835
12.5	1300
25.0	1780
50.0	2140
100.0	2490
200.0	2783
250.0	3108
300.0	3250
350.0	3150
400.0	2967

One-minute incubations at 20° C.
Activities are the average of duplicate determinations.

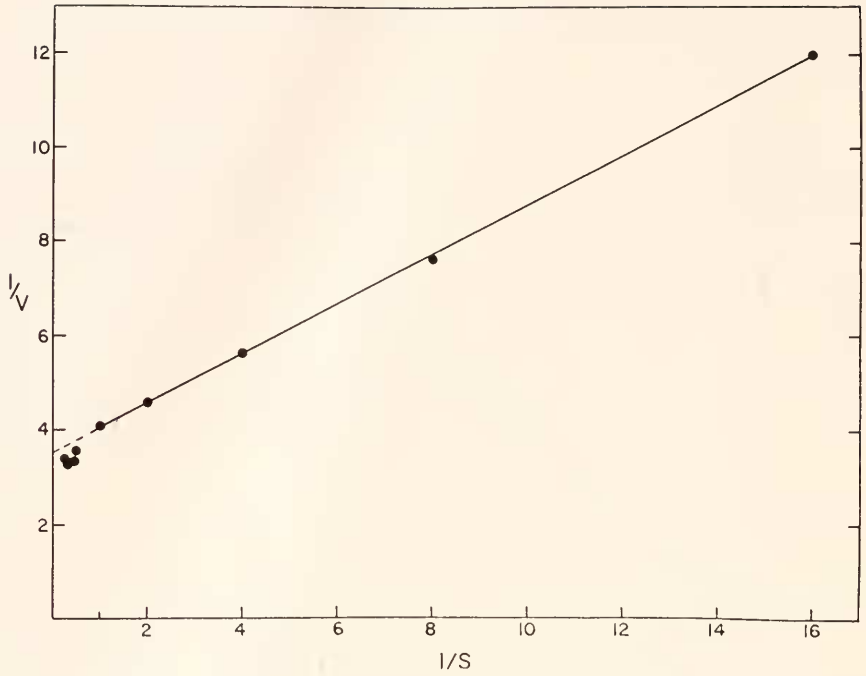

FIGURE 3. Lineweaver-Burk plot of *Lacistorhynchus* urease activity. Velocity reciprocal as (micromoles urea hydrolyzed per gram wet tissue per hour)$^{-1} \times 10^4$. Substrate reciprocal as (millimolar urea)$^{-1} \times 10^2$. One-minute incubations at 20° C. Each point represents the average of duplicate determinations.

the lower substrate concentration range (6.25 m*M* to 100 m*M* urea) (Fig. 3). From 200 m*M* to 400 m*M*, the velocity observed is in excess of that expected.

Activity in saturating substrate concentration

When incubated in the presence of 300 m*M* urea, *Lacistorhynchus* urease activity follows zero-order kinetics for at least four minutes (Fig. 4).

Enzyme stability

When held at 0° C. in the saline solution previously described, *Lacistorhynchus* remain viable for at least 110 hours, the total period of observation. Homogenates freshly prepared from such worms retain full activity (Table IV).

Homogenate preparations *per se* are, however, quite labile; about 93% of the activity is lost in 24 hours at 20° C., and the rate of decay is only little different at 0° C. (Table IV). The loss of specific activity with a concomitant loss of tissue activity is highly suggestive that the decreased activity is due to inactivation and not to general proteolysis.

Storing of whole worms at −20° C. followed by thawing at 0° C. resulted in a loss of activity in homogenates prepared from such material of about 90%; freezing, followed by lyophilization resulted in complete loss of activity.

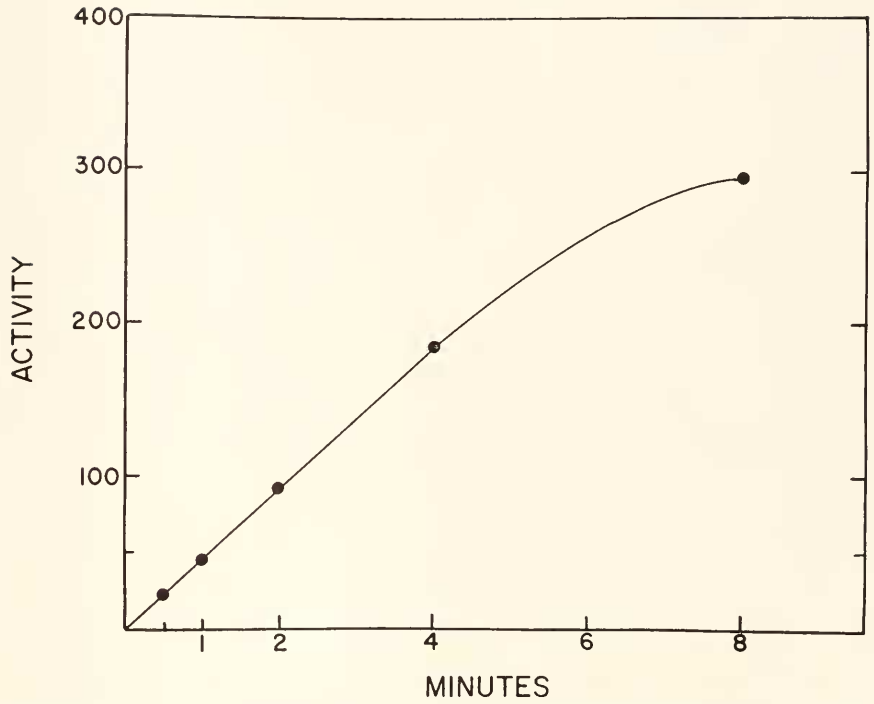

FIGURE 4. Activity of *Lacistorhynchus* urease with time. Incubations at 20° C. Urea concentration 300 m*M*. Activity as micromoles urea hydrolyzed per milligram protein. Each point represents the average of duplicate determinations.

TABLE IV

Stability of Lacistorhynchus urease activity

Material	Holding time (hours)	Holding temperature			
		0° C.		20° C.	
		Tissue activity	Specific activity	Tissue activity	Specific activity
Intact worms	Freshly collected	3450	45.6	—	—
	24	3558	45.6	—	—
	84	3568	45.1	—	—
	110	3336	41.7	—	—
Homogenates	0	3689	43.4	3689	43.4
	1	2329	27.4	1862	21.9
	2	1785	21.0	1360	16.0
	4	1292	15.2	799	9.4
	8	765	9.0	604	7.1
	16	587	6.9	408	4.8
	24	323	3.8	255	3.0

Activities are the average of duplicate determinations from one-minute incubations at 20° C.

C. *Activity exhibited by intact worms*

A single experiment was designed to demonstrate urease activity in intact *Lacistorhynchus*.

One hundred freshly collected worms were divided into four lots of 25 worms each and given a preliminary incubation of three hours at 20° C. in urea-free saline as previously described.

Experimental incubations were performed in respirometer vessels with two sidearms. The main compartment of each vessel contained 1.0 ml. saline with 300 m*M* [^{14}C] urea (California Foundation for Biochemical Research) having a specific activity of 6.67×10^{-3} microcuries per micromole. The center well of each vessel contained 200 microliters 30% KOH for CO_2 capture; sidearm 1 contained 400 microliters of a 1 : 1 (v/v) mixture of 20% TCA and 0.5 *M* lactic acid.

Sidearm apertures were closed with greased, ground-glass stoppers. A worm lot was introduced into the main compartment of each vessel and the mouth of the vessel was immediately closed with a greased rubber stopper, further secured with a rubber band.

TABLE V

Recovery of ammonia and $^{14}CO_2$ following incubation of intact Lacistorhynchus in [^{14}C] urea

Worm group	Wet weight (mgm.)	Acid tip-in (minutes)	Ammonia recovered (micromoles/gm. wet tissue)	$BaCO_3$	
				Mgm.	Radioactivity (counts/min./mgm./gm. wet tissue)
1	155.6	0	11.6	3.3	7,352
2	186.0	5	52.7	3.7	34,559
3	183.2	10	71.0	4.0	39,885
4	170.6	20	102.1	4.6	41,882

See text for experimental details.

The vessels were incubated in a water bath at 20° C. and the acid mixture was tipped in and thoroughly mixed with the contents of the main compartment at desired times. The vessels were allowed to stand 10 hours at room temperature for CO_2 capture, following which period they were opened and the KOH removed from the center well; the latter was rinsed with ten 200-microliter portions of water and these were added to the KOH solution.

For ammonia capture 200 microliters 1 N H_2SO_4 were placed in sidearm 2. One milliliter of a saturated solution of K_2CO_3 was added to the main compartment, the vessels were again sealed, and 24 hours were allowed for diffusion of the ammonia. The H_2SO_4 was removed together with five 200-microliter rinses of the sidearm, diluted to 10.0 ml. and portions were subjected to microdiffusion and ammonia determination as previously described.

Carbon dioxide was precipitated from the KOH solution by the addition of 3.0 ml. of 0.1 N (each) $BaCl_2$-NH_4Cl. The precipitated $BaCO_3$ was centrifuged down at low speed and twice washed with water, and finally plated as a slurry in 70% ethanol on tared stainless steel sample pans (Nuclear-Chicago, SC-12). Radioactivity was determined with a gas flow counter (Nuclear-Chicago, D-47, with "Micromil" window).

Examination of the data obtained, presented in Table V, clearly indicates the production of ammonia and $^{14}CO_2$ from [^{14}C] urea by intact *Lacistorhynchus.* Significant radioactivity of $BaCO_3$ from the sample in which the acid was tipped in at zero-time indicates that the acid mixture is not instantaneously lethal.

The ammonia captured following five minutes' incubation is equivalent to hydrolysis of 316 micromoles urea per gram wet tissue per hour, or about one-tenth the tissue activity of homogenate preparations (Table I).

Because of the high urea concentration employed in the experiment, urea disappearance could not be measured by the Archibald (1945) method; hence no attempt was made to relate urea disappearance and ammonia and $^{14}CO_2$ production.

Discussion

Considerable scepticism has attended the periodic reports of urease in lower animals. This has probably been in part due to the low activities recorded as well as to the inadequacy of experimental procedures used in some instances. Too, doubt regarding the validity of observations on animal urease has probably been strengthened by the fact that activities attributed on numerous occasions to gastrointestinal mucosae are, in fact, of bacterial origin (see Kornberg and Davies, 1955).

In any event, Sumner (1951) offered the opinion that the entire question of animal urease needs reinvestigation, an opinion reiterated by Cohen and Brown (1960) in a more recent review.

The quantity of activity exhibited by the tapeworm urease reported here is of considerable interest. In Table VI the present author has assembled data from a number of other reports of urease of invertebrate origin and compared these with jackbean activity. It is to be understood that because of the widely varying conditions under which these assays were performed, Table VI, of necessity, represents only approximate comparisons. Dry weight values were estimated to be 20% of wet tissue weights when only the latter were available.

It is easily seen that the tissue activity of the tapeworm urease herein reported compares favorably with that of the jackbean material.

The use of antibiotics or other procedures designed to insure bacteriostatic conditions was not resorted to in the present study. Since tapeworms lack a gut, microorganisms would only be present as surface contaminants. It is highly unlikely that the urease activities observed with *Lacistorhynchus* can possibly be attributed to microorganisms. If they were indeed due to surface contaminants, then one might reasonably expect activity to be exhibited in tissue preparations of *Calliobothrium,* a worm parasitizing the same host as *Lacistorhynchus* (Table I). Too, while living worms retain full activity for a considerable period of time, tissue preparations rapidly lose activity, which would be most improbable were the activity of microbial origin (Table IV).

Of great interest to students of parasitism is the fact that cestodes seem to have developed more than one method of adapting to the enormous physiological uremia of their elasmobranch hosts, a condition which Smith (1936) believes to be an archaic biochemical property of this vertebrate class. Among tetraphyllids, urea is apparently not metabolized, but rather is an important osmotic constituent of these worms. Urease activity does not seem to be ubiquitous among trypanorhynchs (Table I), and may not even be characteristic of smaller taxonomic groups within

the Trypanorhyncha, since Dollfus (1942) assigns *Lacistorhynchus* and *Grillotia* to the same family.

Although most previous evidence indicates that adult tapeworms are poikilosmotic animals, consideration of possible osmo-regulation must be given to those forms inhabiting elasmobranch hosts and metabolizing urea (see Read and Simmons, in press).

There are, as yet, no data clarifying the biochemical consequences of urea hydrolysis by *Lacistorhynchus*. Although the hydrolysis of urea is an exergonic reaction, it has never been demonstrated that the free energy available can be trapped for useful work (Cohen and Brown, 1960). The possibility that *Lacisto-*

TABLE VI

Estimated urease activities of various invertebrate species compared with jackbean

Source	Tissue tested	Activity (mgm. urea hydrolyzed per gm. dry weight per 5 mins.)	Temperature (degrees Centigrade)	pH	Approximate concentration of urea (molar)	Author
Jackbean	Meal (not defatted)	486–735	20	7.0	0.250	Damodaran and Sivaramakrishnan (1937)
Cestodes						
Lacistorhynchus tenuis	Whole	70	20	7.0	0.400	Present
Pterobothrium lintoni	Whole	700	20	7.0	0.400	Present
Nematodes						
Ascaris lumbricoides	Intestine	0.73	?	6.0	0.007	Savel (1955)
	Muscle	0.28	?	6.0	0.007	Savel (1955)
Nematodirus spp.	Whole	0.036	?	7.3	0.008	Rogers (1952)
Sipunculid						
Sipunculus nudus	Intestine	0.30	?	?	0.0013	Florkin and Duchateau (1942)
Annelid						
"*Lumbricus agricola*"	Whole	0.0007	?	?	0.167	Przylecki (1922)
Molluscs						
Mytilus edulis	Whole	0.009	?	?	0.167	Przylecki (1922)
Helix pomatia	Whole	0.014	?	?	0.167	Przylecki (1922)
Arthropods						
Lucilia sericata	Maggots reared aseptically	1.2	35–36	7.4	?	Robinson and Baker (1939)
Astacus fluviatilis	Whole	0.001	?	?	0.167	Przylecki (1922)

rhynchus might further utilize at least part of the ammonia and carbon dioxide produced by its urease must be considered. Campbell (1960) demonstrated the formation of α-[1-^{14}C] alanine and, probably, [^{14}C] succinic acid from the degradation of [2-^{14}C] uracil by the rat cestode, *Hymenolepis diminuta*, for which the most tenable explanation was a mechanism of carbon dioxide fixation; Daugherty (1954) has reported the synthesis of amino nitrogen from ammonium carbonate by the same species.

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Summary

1. Strong urease activity was measured in tissue preparations of two species of trypanorhynch cestodes; activity was not detected in seven species of tetraphyllids, nor in a third trypanorhynch.

2. Tissue activity of *Lacistorhynchus tenuis* homogenates was in excess of 3000 micromoles urea hydrolyzed per gram wet tissue per hour; that of *Pterobothrium lintoni* was about ten times greater. Tissue activity of intact *Lacistorhynchus* was about one-tenth that of homogenate preparations.

3. The worm activity was partially characterized in homogenate preparations.

4. Living worms retain full activity for at least 110 hours, but homogenates rapidly decline in activity; less than 10% of the original activity is measured after 24 hours.

5. Production of ammonia and of $^{14}CO_2$ from [^{14}C] urea was demonstrated using intact *Lacistorhynchus*.

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